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QTL mapping of physiological traits associated with salt tolerance in *Medicago truncatula* Recombinant Inbred Lines

Soumaya Arraouadi [a,*], Mounawer Badri [b], Chedly Abdelly [b], Thierry Huguet [c], Mohamed Elarbi Aouani [d]

[a] Laboratory of Legumes, Centre of Biotechnology of Borj Cedria, B.P. 901, 2050 Hammam-Lif, Tunisia
[b] Laboratory of Plant Extremophyl, Centre of Biotechnology of Borj Cedria, B.P. 901, 2050 Hammam-Lif, Tunisia
[c] Laboratoire de Symbiose et Pathologie des Plantes, INP-ENSAT, B.P. 107, 31326 Castanet Tolosan Cedex, France
[d] NEPAD/North Africa Biosciences Network, National Research Centre, El Buhouth St, Cairo, 12311, Egypt

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ABSTRACT

In this study, QTL mapping of physiological traits in the model Legume (*Medicago truncatula*) was performed using a set of RILs derived from LR5. Twelve parameters associated with Na^+ and K^+ content in leaves, stems and roots were measured. Broad-sense heritability of these traits was ranged from 0.15 to 0.83 in control and from 0.14 to 0.61 in salt stress. Variation among RILs was dependent on line, treatment and line by treatment effect. We mapped 6 QTLs in control, 2 in salt stress and 5 for sensitivity index. No major QTL was identified indicating that tolerance to salt stress is governed by several genes with low effects. Detected QTL for leaf, stem and root traits did not share the same map locations, suggesting that genes controlling transport of Na^+ and K^+ may be different. The maximum of QTL was observed on chromosome 1, no QTL was detected on chromosomes 5 and 6.

1. Introduction

Legumes are important economic crops that provide humans with food, livestock with feed, and industry with raw materials [1]. The genus *Medicago* contains 83 species, including alfalfa (*Medicago sativa*), that are typically either tetraploid perennial or diploid annual species [2,3]. Properties shared by the majority of legume species are the mutualistic interactions with nitrogen-fixing rhizobia and symbiotic mycorrhizal fungi [4,5]. Consequently they do not need costly and polluting chemical nitrogen fertilizers [6]. Salinity is one of major stresses limiting crop production worldwide, affecting near 40% of agricultural lands located in arid and semi-arid climates [7]. Indeed, salt can impose a multifaceted injury to *Medicago* genus plants, such as seed germination, vegetative growth, and yield. *Medicago truncatula* is widely used as a model plant for legume genetics and genomics [8], by virtue of being an annual, diploid and autogamous legume with a moderate genome size (500–550 Mbp). *M. truncatula* is found in a variety of edaphic and bioclimatic conditions in Tunisia, suggesting that genotypes adapted to local biotic and abiotic stresses could be identified in natural populations and integrated into breeding programs. It is now recognized that tolerance of salinity by higher plants, in common with other environmental stresses, is genetically and physiologically complex, and that salt affects numerous plant processes at all levels of organization.

At the very least, ion transport, selectivity, excretion, nutrition, and compartmentation are involved, together with growth, water use and water use efficiency. Salt tolerance of the crop is the final manifestation of several components, such as Na^+ content, K^+ content, ion balance and ion compartmentation, etc. To keep the Na^+ level low inside a plant cell is not an easy task especially when the external Na^+ levels are high in saline soils. A major toxic ion from saline soil is Na^+ that gets into plant cells through Na^+ permeable transporters [9]. In spite of a large inter and intraspecific variability of legume tolerance to salt, these species adopt generally the exclusive strategy, characteristic of sensitive plants. Legumes tend to restrict Na^+ transport towards shoots and, thus, maintain relatively low salt levels in their photosynthetic organs [10]. Therefore, salt tolerance has often been found to be associated with lower accumulation of sodium (Na^+) in the shoot [11,12] but not always [13]. The inter-specific comparison of some *Medicago* species showed that in *M. arborea* (salt sensitive species) the highest Na^+ concentrations were observed in the leaf blades, whereas *M. citrina* (salt tolerant species) distributed the salt in the petioles [14]. Numerous studies [15,16] tried to dissect a complex physiological trait of salt tolerance using improved methods of identifying and measuring physiological components such as shoot sodium concentration, plant survival scores and plant vigor. However, the development of molecular markers has made genetic analysis possible to investigate quantitative inheritance; that it shows continuous variation and a high degree of environmental sensitivity [17]. The identification of some of

Abbreviations: QTL, Quantitative traits loci; RILs, Recombinant Inbred Lines; LR5, A cross between the tolerant line Jemalong A17 and susceptible line F83005.5.

* Corresponding author. Fax: +216 79 325 728.
E-mail address: bio.soumaya@gmail.com (S. Arraouadi).

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the quantitative trait loci (QTLs) that contribute to natural variation in salt tolerance should be instrumental in eventually manipulating the perception of salinity and the corresponding responses. Therefore, Na^+ and K^+ uptake, balance and distribution within the cell and the plant have been considered of great interest for quantitative trait loci (QTL) analysis in other crops such as citrus [18], rice [19] and durum wheat [20]. It is quite possible that the QTL for Na^+:K^+ ratio, which was independent of the QTL for sodium or potassium uptake per se, reflects selectivity by membrane-based transport systems.

Actually, based on the QTL mapping in multiple related populations derived from two parents, a maximum likelihood estimation method was proposed, which can incorporate several populations derived from three or more parents and also can be used to handle different mating designs [21]. In this last years, many studies of QTL analyses for agronomical traits of interest have been carried out in *M. truncatula* such as tolerance to drought stress [22], aerial morphogenesis [23], flowering date [24], resistance to *Aphanomyces euteiches* [25], seed mineral concentration and content [26], salt tolerance [27], seed germination and pre-emergence growth at extreme temperatures [28] and in water deficit [29].

The study reported in this paper aims to identify and map QTL associated with Na^+ and K^+ contents in the leaves, stems and roots controlling salt tolerance in *M. truncatula* using Jemalong A17 × F83005.5 RILs population (LR5). We map and discuss several loci explaining the variability of these physiological traits in 0 and 50 mM NaCl conditions, at a vegetative stage.

2. Results

2.1. Phenotypic variation and correlations among traits

Analysis of variance revealed that variation between the RILs for leaf, stem and root dry weights and their concentrations of Na^+ and K^+ was dependent on line, treatment, and line by treatment interaction effects (Table 1). Salt stress treatment explained most of the trait variation. This finding indicates a difference among the RILs in the responses to environmental conditions.

In control conditions, fewer values were found for F83 line for StNa, LeaNa, RoNa, StK, LeaK, RoK, StNaK, LeaNaK, and RoNaK, while the largest values were for JA17 for StNa, LeaNa, RoNa, StNaK, LeaNaK and RoNaK traits under salt-stressed conditions.

To estimate the importance of measured traits in the description of the observed phenotypic variability between analyzed RILs, we measure broad-sense heritability (H^2) of these traits. Biomass traits showed higher broad-sense heritability (H^2) in control than in salt treatment, and heritability was higher for StDW and LeaDW. Furthermore, heritability values for traits relative to stems, leaf and root Na^+ and K^+ uptake are low to moderate.

Proprieties of the trait distribution were estimated by ANOVA analysis over 4 replicates. According to Kurtosis and Skewness estimation trait distribution reflects the variability observed between studied lines. Physiological values in the whole population show a relatively normal distribution around a population mean that lies between the parental values. Some RILs had more extreme values than the parental lines showing a transgressive segregation; this is obvious for all traits under control and salt-stressed conditions (Table 2).

Correlations between measured traits showed 29 and 39 of 72 possible correlations were significant in control and 50 mM NaCl conditions, respectively (Table 3). Fourteen (14) of these 29, and 17 of the 39 correlations were positive. Our results showed that the sign and level of correlations between measured traits were generally influenced by NaCl stress application. Indeed, some traits showed significant correlations in control which become highly significant in NaCl treatment, and there are ones which explain contrary. A strong positive correlation was observed between leaf, stem and root dry weights. Negative associations were observed between dry weights (leaves, stems and roots) with Na^+ and K^+ contents. On the other hand, Na^+ and K^+ concentrations in leaves and stems were positively correlated.

2.2. QTL mapping

QTL mapping results are summarized in Table 4, where the name of QTL contains the trait name suffixed with the type of treatment and an ordering number from the first chromosome. A total of 13 QTLs on 8 linkage groups were detected for measured traits in control and salt treatments. Six of these QTLs were identified in control treatment, two in salt stress conditions and five relative to the sensitivity index. The percentage of phenotypic variance explained by a single QTL (R^2) ranged from 9.5 to 12.5% of the phenotypic variation. We mapped three QTLs for root Na^+/K^+ ratio concentration under control treatment on chromosomes 1 and 8, and one QTL for leaf Na^+/K^+ under salt conditions on chromosome 1. One QTL for stem Na^+ concentration under control conditions was detected on chromosome 2, one QTL for stem Na^+ total quantity under control conditions was on chromosome 3, and one QTL for leaf Na^+ total quantity under control conditions was mapped on chromosome 1. One QTL has been identified for root K^+ total quantity under salt condition on chromosome 8. Furthermore, five QTLs relative to sensitivity index were detected, which one for stem K^+ concentration identified on chromosome 4, three for leaf K^+ concentration were mapped on chromosomes 1 and 2 and one for root K^+ concentration detected on chromosome 7. Six QTLs were detected on chromosome 1, two QTLs were on each chromosome 2 and 8 and one QTL on each chromosome 3, 4 and 7. No QTL was found on chromosomes 5 and 6. The chromosome 1 seemed to be highly involved in the genetic variation of leaf and root Na^+/K^+ ratio concentration between RILs of LR5 under control and salt-stressed conditions. Overall, no major QTL was identified for measured traits which may be due to the fact that many genes with small effects segregate in this population.

For an easier overview of overlapping QTL between traits and growth conditions, QTL regions are illustrated in Fig. 1. Both parental lines contributed to the expression of the different target traits. No overlapping QTLs was found for measured traits under control and salt-stressed conditions (Table 4 and Fig. 1), except those relative to leaf Na^+/K^+ ratio concentration, leaf Na^+ total quantity and leaf K^+ concentration sensitivity index traits on chromosome 1. Among the six QTLs detected under control conditions, no QTL was mapped under salt-stressed conditions.

3. Discussion

3.1. Phenotypic variation and correlations among traits

The line by treatment effect on stem and leaf dry weights, and root Na^+/K^+ content suggests that the relative performance of genotype (especially RILs) changed depending on environmental conditions. This finding makes assessing the causal relationship between genotype and phenotype difficult. Similarly, this result was also reported for a RIL population of rice (Bala × Azucena mapping population) where a QTL × environment interaction was observed [30]. The effect of environmental conditions has to be tested on a range of genotypes, as genotypes × environment interactions are likely to be detected. Genotype × environment interaction is a common characteristic for quantitative traits, and has been a subject of great concern for breeding programs. The marker assisted selection is generally more efficient than phenotypic selection in the presence of genotype × environment interaction [31]. Thus, the challenge is to define phenotypes that are a true reflection of the genotypic differences, and to find the right genes/phenotypes that work well in target environments.

Table 1
Proportions and significance levels of line, treatment, and line by treatment interaction effect, and heritabilities (H^2) for measured traits under control and salt-stressed conditions.

		StDW	LeaDW	RoDW	StNa	LeaNa	RoNa	StK	LeaK	RoK	StNaK	LeaNaK	RoNaK
Line	F	2.90***	3.56***	1.15^{ns}	0.89^{ns}	0.91^{ns}	1.00^{ns}	1.23^{ns}	1.29*	1.08^{ns}	0.85^{ns}	0.93^{ns}	1.25*
	%	0.61	2.19	3.05	0.34	0.14	0.50	5.10	3.44	3.10	0.59	0.17	0.28
Treatment	F	470.53***	156.49***	35.25***	258.41***	632.48***	199.43***	21.96***	35.15***	32.76***	142.72***	545.53***	443.54***
	%	98.89	96.36	93.75	99.31	99.73	99.22	91.38	94.02	93.73	98.73	99.67	99.43
Line × treatment	F	2.39***	2.34***	1.20^{ns}	0.90^{ns}	0.81^{ns}	0.58^{ns}	0.85^{ns}	0.95^{ns}	1.11^{ns}	0.98^{ns}	0.85^{ns}	1.29*
	%	0.50	1.44	3.20	0.35	0.13	0.29	3.52	2.54	3.17	0.68	0.16	0.29
H^2													
Control	σ^2_g	0.012	0.016	0.002	11,930,000	1,766,000	2,661,000	0	4,998,000	18,080,000	0	0.002	0.029
	σ^2_ε	0.014	0.014	0.027	71,660,000	14,130,000	29,360,000	262,400,000	100,100,000	76,980,000	0.281	0.017	0.108
	H^2 (control)	0.77	0.82	0.23	0.40	0.33	0.27	0.00	0.17	0.48	0.00	0.32	0.52
NaCl	σ^2_g	0.001	0.003	0.001	13,460,000	3,411,000	8,886,000	25,270,000	3,697,000	0	0.021	0.006	0.025
	σ^2_ε	0.003	0.007	0.019	269,100,000	23,720,000	233,400,000	149,200,000	60,150,000	361,400,000	0.401	0.057	0.697
	H^2 (NaCl)	0.57	0.63	0.17	0.17	0.37	0.13	0.40	0.20	0.00	0.17	0.30	0.13

Percentages (%) given in the table were calculated from variance analysis. F: Snedecor–Fisher coefficient. Significance levels; ns: not significant ($P > 0.05$), *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. σ^2_g: genetic variance, σ^2_ε: environmental variance. Stem dry weight (StDW, g), leaf dry weight (LeaDW, g), root dry weight (RoDW, g), stem K^+ concentration (StK, μmol mg^{-1} of stem dry weight), leaf K^+ concentration (LeaK, μmol mg^{-1} of leaf dry weight), and root K^+ concentration (RoK, μmol mg^{-1} of root dry weight), stem Na^+ concentration (StNa, μmol mg^{-1} of stem dry weight), leaf Na^+ concentration (LeaNa, μmol mg^{-1} of leaf dry weight), root Na^+ concentration (RoNa, μmol mg^{-1} of root dry weight), stem Na^+/K^+ concentration ratio (StNaK), leaf Na^+/K^+ concentration ratio (LeaNaK), and root Na^+/K^+ concentration ratio (RoNaK).

All the trait distributions were continuous and had a normal distribution in the control and NaCl treatments, indicating polygenic segregation or monogenic segregation with a high error effect. All measured traits for RILs showed significant transgressive segregations with values either larger or smaller than those of the parents. Similarly, of the transgressive segregation observed at the phenotypic level of studied population, significant transgressive segregations were reported for physiological traits in rice [19], and for morphological traits in *Arabidopsis* under contrasting nitrate availability in the soil [32], in sunflower under drought stress [33], and in durum wheat under water deficit [34].

Heritabilities estimation of measured traits relative to dry weight, showed higher values (H^2) in control than under salt-stressed conditions (Table 1). But traits relative to leaf, stem and root Na^+ and K^+ contents seemed to be moderately heritable. This finding is due to a higher genetic variance for StDW, LeaDW and RoDW under control conditions and to a higher environmental variance for the remaining traits under salt treatment. Similarly, this result was also mentioned under drought conditions and many studies found moderate heritability of yield [35].

Our results showed that NaCl stress application influences most of negative correlations between measured traits under control and salt-stressed conditions. A strong positive correlation was observed between leaf, stem and root dry weights. The positive correlations found between ion concentration ratio in root and stem, leaves and root dry weights suggest that tolerant lines are those who are able to maintain low levels of root Na^+/K^+ concentration ratio in their leaves. Similarly, shoot and root dry weight showed low correlation with Na^+, K^+ concentration and Na^+/K^+ ratio in rice (*Oryza sativa* L) in salt conditions [36]. Indeed, this suggestion supports the notion that excess Na^+ was the primary cause of salt sensitivity in non-halophytes [12]. Moreover, shoot Na^+ accumulation and salt tolerance were not correlated in bread wheat, assessing that salinity tolerance would be identified by tissue tolerance [37].

On the other hand, this observed variability of salt stress response between studied lines able us to understand salt stress response by mapping QTL which can identify chromosomes regions which could be responsible. Many studies focus on salt tolerance of this species, like study which compared the differential root growth of two genotypes of *M. truncatula* (108-R and Jemalong A17) in response to salt stress. Jemalong A17 was more tolerant to salt stress than 108-R, where it grew well at high salinity levels (120 mM NaCl) [38]. Transcription factor gene after salt stress in *M. truncatula* roots was analyzed for this line and it was suggested that spatial differences of transcription factor gene regulation by environmental stresses in various root regions may be crucial for the adaptation of their growth to specific soil environments [39].

3.2. QTL mapping

A moderate number of QTL on 6 of the 8 linkage groups were detected for leaf, stem and root Na^+ and K^+ uptake in control and salt-stressed conditions. Nevertheless, this work gives access to an interesting part of the genetic variation of salt-stressed response in the JA17 × F83 population. Furthermore, most of detected QTLs were mapped under control treatment. By contrast, more QTLs were detected for morph-physiological traits related to drought tolerance in rice (*O. sativa*) under stress conditions [40]. Similarly, among the 24 QTLs detected under well-watered treatment, 5 (about 21%) were also detected under water-stressed conditions in sunflower [33]. In the present study, among the 6 QTLs detected under control treatment, no QTL was detected under salt-stressed conditions (Table 4). We postulate that the loci that are not stable across treatments reflect adaptation to this constraint. Indeed, the comparison between QTLs obtained in control and stressed treatments could allow the distinction between constitutive and adaptive behaviors [41,33,40]. In

Table 2
Dry weight and stem, leaf and root Na^+, K^+ content in the LR5 population (JemalongA17 × F83005.5) of *M. truncatula* under control and salt-stressed conditions.

Traits	Treatment	Parental lines		Recombinant Inbred Lines				
		JemalongA17	F83005.5	Means	Std dev	Range	Skewness	Kurtosis
StDw	Control	0.52	0.39	0.34	0.13	0.11–0.87	1.18	1.79
LeaDw		0.64	0.61	0.43	0.14	0.18–1.00	0.93	0.91
RoDw		0.37	0.38	0.28	0.12	0.09–0.86	4.53	34.17
StNa		12,990.37	11,776.15	12,165.03	4028.13	6132.54–44,547.92	9.85	130.26
LeaNa		9282.45	9642.83	10,456.21	2907.42	1858.68–20,876.95	1.86	6.28
RoNa		12,858.35	17,300.92	11,671.9	4054.02	4354.61–30,121.38	1.49	4.65
StK		34,386.72	38,980.97	40,517.08	10,513.82	3432.27–89,789.85	14.17	234.11
LeaK		27,884.13	28,315.07	34,151.93	7192.19	12,414.37–64,354.81	1.82	4.67
RoK		17,646.07	17,848.14	22,229.48	7339.88	9060.25–54,782.95	0.85	1.79
StNaK		0.39	0.31	0.32	0.13	0.09–1.28	14.17	234.11
LeaNaK		0.34	0.34	0.32	0.09	0.07–0.63	1.82	4.67
RoNaK		0.74	0.97	0.56	0.31	0.30-3.63	11.44	189.24
StDw	NaCl	0.15	0.14	0.17	0.05	0.06–0.39	0.48	0.65
LeaDw		0.29	0.3	0.32	0.07	0.15–0.63	0.12	−0.16
RoDw		0.2	0.19	0.2	0.09	0.05–0.76	5.8	57
StNa		27,206.21	36,965.03	34,755.88	8963.96	17,120.43–91,809.97	10.45	169.99
LeaNa		17,505.73	19,317.09	30,239	4817.44	18,697.85–43,762.52	0.76	0.96
RoNa		28,847.62	29,196.99	17,669.47	5000.36	4697.36–36,652.87	3.72	31.02
StK		31,926.31	44,183.56	29,922.32	10,192.91	16,884.80–126,200.44	9.76	139.16
LeaK		25,460.79	37,471.41	21,620.34	3355.21	12,613.90–35,851.27	1.8	7.03
RoK		17,412.34	18,496.96	26,237.02	8885.4	7665.11–81,465.87	12.86	221.15
StNaK		0.85	0.83	0.94	0.41	0.41–4.36	9.76	139.16
LeaNaK		0.69	0.52	0.75	0.16	0.42–1.24	1.8	7.03
RoNaK		1.74	1.63	1.64	0.57	0.78–6.28	4.82	42.87

Std dev: standard deviation. Stem dry weight (StDW, g), leaf dry weight (LeaDW, g), root dry weight (RoDW, g), stem K^+ concentration (StK, μmol mg^{-1} of stem dry weight), leaf K^+ concentration (LeaK, μmol mg^{-1} of leaf dry weight), and root K^+ concentration (RoK, μmol mg^{-1} of root dry weight), stem Na^+ concentration (StNa, μmol mg^{-1} of stem dry weight), leaf Na^+ concentration (LeaNa, μmol mg^{-1} of leaf dry weight), root Na^+ concentration (RoNa, μmol mg^{-1} of root dry weight), stem Na^+/K^+ concentration ratio (StNaK), leaf Na^+/K^+ concentration ratio (LeaNaK), and root Na^+/K^+ concentration ratio (RoNaK).

accordance with [19], our results showed that detected QTLs for traits measured on the leaves and the roots did not share the same map locations, suggesting that the genes controlling the transport of Na^+ and K^+ between the leaves and the roots may be different or induced uncoordinatedly by salt stress. The processes of Na^+ and K^+ uptake in rice were considered to be independent under salt stress [42]. The uptake of Na^+ and K^+ maybe be independent [17], due to the major pathways of Na^+ and K^+ uptake in rice occur in parallel and not directly in competition. K^+ is detrimental for Na^+ plant tolerance due to the fact that K^+ and Na^+ are chemically very similar. Other studies, reported that *SOS* (for salt overly sensitive) genes, *SOS1*, *SOS2* and *SOS3* in *Arabidopsis*, were postulated to encode regulatory components controlling plant K^+ nutrition which in turn was essential for salt tolerance, and considered that K^+ nutrition correlated closely with salt tolerance in salt stress [43].

In this study we identified also five QTLs according to the sensitivity index for only K^+ content in leaves, stems and roots. This result confirms the importance of this ion in plant growth. However, plants need a small amount but high concentration of K^+ for specific functions in the cytoplasm and a major portion (~90%) of it is localized in vacuoles, where it acts as an osmoticum [44]. On the other hand, no major QTL was identified for measured traits which may be due to the fact that many genes with small effects segregate in this population. Similarly, complex physiological traits have on recent occasions been described by a small number of major QTLs [19,15]. The QTLs identified in the present study underlined that several putative genomic regions are involved in the response of leaves, stem and roots Na^+ and K^+ uptake traits under control and salt-stressed treatments (Table 4). Accordingly, numerous studies have reported the polygenic determinism of shoot and stem Na^+ and K^+ concentrations in rice [17,19], in tomato [15], in *Arabidopsis* [45], and in cereals [46]. This polygenic feature of plant's response to salt stress makes it difficult to transfer individual genes using traditional plant breeding or marker-assisted selection (MAS).

Most of the identified QTLs did not share the same locations on the LR5 genetic map suggesting their adaptation to this constraint. The sensitivity to environmental conditions may be due to the responsiveness of regulation (e.g. transcription) of the QTL gene to an environmental cue. Our results will provide important information for further functional analysis of salt-tolerance genes in *M. truncatula*. Detailed characterization of the QTLs regions through the development and evaluation of near-isogenic lines will lead to an improved understanding of salt tolerance and might set the stage for the positional cloning of salt tolerance genes. As the QTLs found here are relatively weak and they were identified from greenhouse study, their usefulness should therefore be evaluated under field condition, and also validated in other genetic backgrounds.

In the light of our previous results on QTLs mapping for morphological traits of LR5 population [27], few common QTLs, with this study, were identified for measured traits under salt stress on chromosomes 3 and 8, suggesting that genetic bases for tolerance to both traits could be different. The high number of QTLs for physiological and morphological traits was detected on chromosomes 1 and 8. Furthermore, QTLs found for LR5 population related to final percentage of germination and for early embryonic axis elongation rate for LR4 in chromosome 3 [28] at respectively sub-optimal and supra optimal temperatures, are co-located with those identified in this study concerning stem Na^+ total quantity and length of orthotropic axis in [27]. It remains to be investigated whether the co-locations of these QTLs are caused by the presence of closely linked genes or by pleiotropic effects from the same genes on these traits.

4. Material and methods

4.1. Plant material and experimental conditions

A segregating population LR5 of Recombinant Inbred Lines (133 RILs) of *M. truncatula* at F_8 generation derived from a cross between Jemalong A17 (JA17) and F83005.5 (F83) was used. RILs were developed by single-seed descent until the F_8 generation at the INP-ENSAT, France. The two parents were included in all experiments. Seed germination was performed using mechanic scarification and NaClO 12% as agent for seed surface sterilization. The soaked seeds have been sown in Petri dishes on 0.9% agar medium before being

Table 3
Estimated correlations between measured traits for RILs of LR5 of *M. truncatula* under control and salt-stressed conditions.

	StDW	LeaDW	RoDW	StNa	LeaNa	RoNa	StK	LeaK	RoK	StNaK	LeaNaK	RoNaK
Control treatment												
StDW	1.000											
LeaDW	0.741***	1.000										
RoDW	0.415***	0.319***	1.000									
StNa				1.000								
LeaNa	−0.159**			0.195***	1.000							
RoNa	−0.115*		−0.371***			1.000						
StK	−0.237***	−0.133**		0.225***			1.000					
LeaK		−0.127*			0.246***	−0.182***	0.224***	1.000				
RoK	−0.352***	−0.297***	−0.500***			0.660***			1.000			
StNaK				0.340***			−0.196***			1.000		
LeaNaK	−0.133**				0.700***		−0.146**	−0.392***	0.105*		1.000	
RoNaK	0.147**	0.178***				0.436***			−0.188***			1.000
NaCl treatment												
StDW	1.000											
LeaDW	0.753***	1.000										
RoDW	0.282***	0.313***	1.000									
StNa	−0.161**	−0.223***		1.000								
LeaNa	−0.349***	−0.374***	−0.212***	0.232***	1.000							
RoNa			−0.269***	−0.125*		1.000						
StK	−0.244***	−0.326***		0.277***	0.196***	−0.126*	1.000					
LeaK	−0.127*	−0.132**			0.357***		0.149**	1.000				
RoK	−0.148**	−0.146**	−0.370***			0.561***			1.000			
StNaK				0.741***			−0.292***			1.000		
LeaNaK	−0.212***	−0.226***	−0.183***	0.202***	0.625***			−0.428***		0.151**	1.000	
RoNaK	0.189***	0.175***	0.103*			0.364***			−0.313***	−0.147**		1.000

Significance levels; *$P<=0.05$, **$P<=0.01$, ***$P<=0.001$. Stem dry weight (StDW), leaf dry weight (LeaDW), root dry weight (RoDW), stem Na^+ concentration (StNa), leaf Na^+ concentration (LeaNa), root Na^+ concentration (RoNa), stem K^+ concentration (StK), leaf K^+ concentration (LeaK), root K^+ concentration (RoK), stem Na^+/K^+ concentration ratio (StNaK), leaf Na^+/K^+ concentration ratio (LeaNaK), and root Na^+/K^+ concentration ratio (RoNaK).

vernalized at 4 °C for 72 h. Once the emerging root attained a length of 4 mm, seedlings were individually transferred to 33 centiliters pots (8 cm diameter and 10.5 cm deep) filled with sterilized sand using chlorhydric acid 0.05%. Each plant was grown in an individual pot in greenhouse in controlled conditions with a temperature of 25 °C, a relative humidity of 80% and a photoperiod of 16/8 h. Four replicates per line and per treatment were used. The experimental design was completely randomized. During 60 days, the plants were irrigated 2 times per week. For control treatment, we used a nutritive solution as described by [47] whereas the iron source was modified by adding Fe-EDTA. In salt stress treatment, we added 50 mM of NaCl to the nutritive solution. Salt stress was applied at seedling stage directly after germination. For both treatments, the whole retention capacity was maintained by weighting pots and adding the nutritive solution to compensate the decrease in volume. To overcome NaCl accumulation problem in the substrate, sand in the pots was washed by distilled water 2 times per week.

4.2. Trait measurement

The harvest of plants was performed 60 days after the beginning of the experiment. At harvest, we measured seventeen quantitative and physiological traits of the stem dry weight (StDW), leaf dry weight (LeaDW), root dry weight (RoDW), stem Na^+ concentration (StNaC), stem Na^+ total quantity (StNaTQ), leaf Na^+ concentration (LeaNaC), leaf Na^+ total quantity (LeaNaTQ), root Na^+ concentration (RoNaC), root Na^+ total quantity (RoNaTQ), stem K^+ concentration (StKC), stem K^+ total quantity (StKTQ), leaf K^+ concentration (LeaKC), leaf K^+ total quantity (LeaKTQ), root K^+ concentration (RoKC), root K^+ total quantity (RoKTQ), Na^+/K^+ concentration ratio (Na^+/K^+C), and Na^+/K^+ total

Table 4
Map positions and genetic effect of putative QTLs detected for measured traits in Recombinant Inbred Lines of *M. truncatula* in control and 50 mM of NaCl treatment.

Traits	Treatment	QTLs[a]	Linkage group	Left marker	QTL position and confidence interval (cM)	LOD	Effect[b]	R^2 (%)
Stem Na^+ concentration	Control	StNaCct.2	II	MTE14	42 (40–46)	3.63	2090.559	11.9
Root Na^+/K^+ concentration ratio	Control	RoNaKCct.1	I	MTE77	54 (48–56)	3.02	0.156	10.0
		RoNaKCct.1	I	MTE6	66 (64–70)	2.85	−0.154	9.5
		RoNaKCct.1	VIII	MTE94	34 (26–46)	2.88	−0.105	9.6
Stem Na^+ total quantity	Control	StNaTQct.3	III	MTE19	26 (22–32)	2.93	60.838	9.7
Leaf Na^+ total quantity	Control	LeaNaTQct.1	I	MTE75	10 (4–14)	3.22	44.798	10.8
Leaf Na^+/K^+ concentration ratio	NaCl	LeaNaKCsl.1	I	MTE4	12 (4–24)	2.98	0.055	9.8
Root K^+ total quantity	NaCl	RoKTQsl.8	VIII	MTE94	26 (20–34)	3.01	−58.034	9.9
Stem K^+ concentration	Sensitivity Index	StKCsi.4	IV	MTE29	50 (42–68)	3.82	−11.274	12.5
Leaf K^+ concentration	Sensitivity Index	LeaKCsi.1	I	MTE75	10 (6–20)	3.49	−10.190	11.7
		LeaKCsi.1	I	MTE88	78 (74–78)	2.90	13.214	9.6
		LeaKCsi.2	II	MTE72	50 (48–56)	3.54	15.215	11.7
Root K^+ concentration	Sensitivity Index	RoKCsi.7	VII	MTE69	46 (36–54)	3.08	12.807	10.2

[a] The name of QTL contains the trait name suffixed with the type of treatment and an ordering number from the first linkage group, Control (ct), NaCl treatment (sl), Sensitivity index (si).
[b] Effect of Jemalong A17 allele for Jemalong A17 x F83005.5 population.

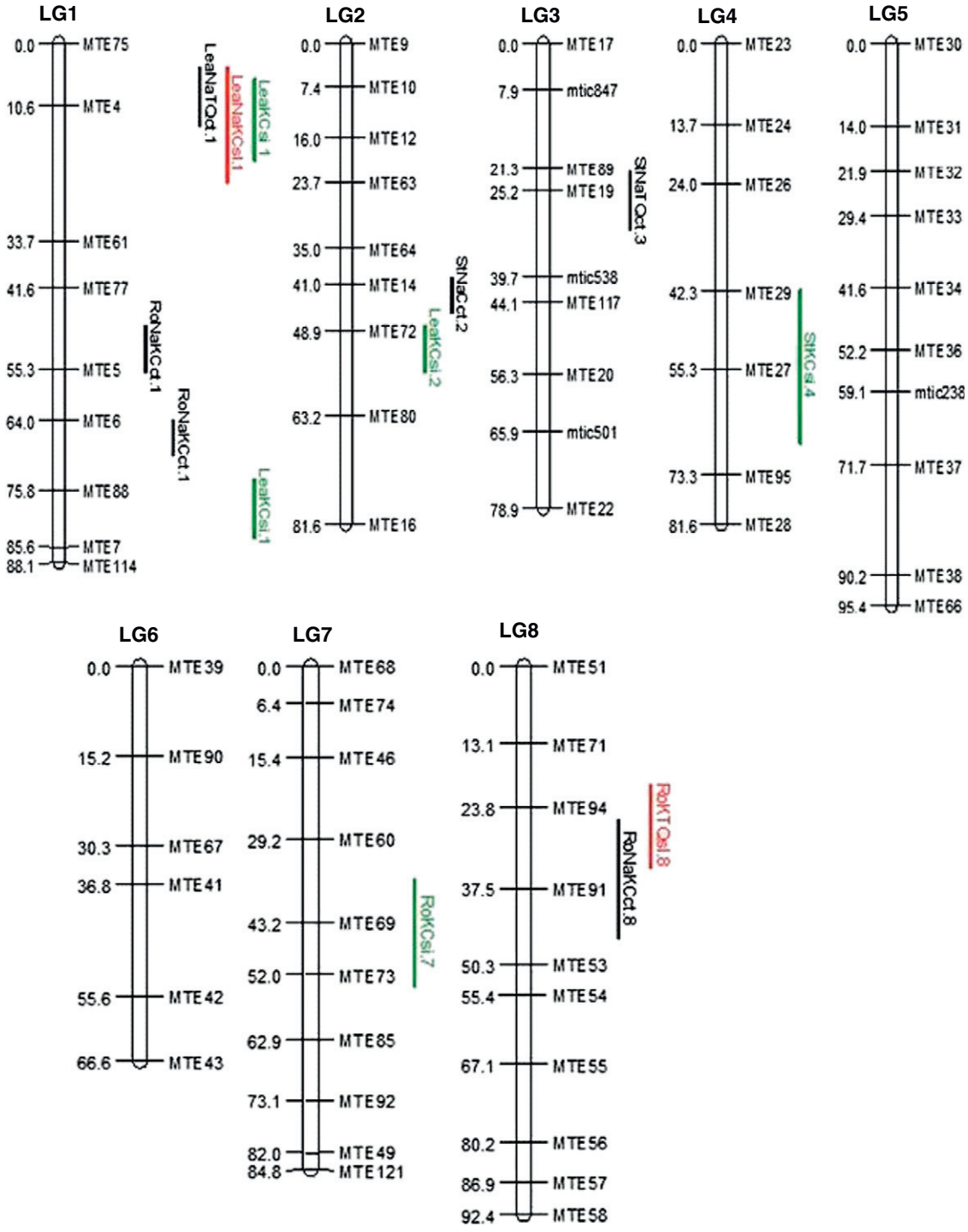

Fig. 1. The Jemalong A17 × F83005.5 genetic map of eight linkage groups (LG) showing the location of putative QTLs of physiological measured traits in control (colored in black), in stressed conditions (colored in red) and for sensitivity index (colored in green). The QTLs were designated for all measured traits, followed by "ct", "sl" and "si" for control, salt stressed conditions or sensitivity index, respectively, and followed by the corresponding linkage group number. The lengths of the arrows indicate the 2-LOD support intervals. Stem Na^+ concentration (StNaC), stem K^+ concentration (StKC), Leaf K^+ concentration (LeaKC), root K^+ concentration (RoKC), root Na^+/K^+ concentration ratio (RoNaKC), stem Na^+ total quantity (StNaTQ), leaf Na^+ total quantity (LeaNaTQ), leaf Na^+/K^+ concentration ratio (LeaNaKC), and root K^+ total quantity (RoKTQ).

quantity ratio (Na^+/K^+ TQ). Fresh weights of each part of each plant were measured at time of harvest. Plants were incubated in drying oven during 5 days in 60 °C, and then dry weights of leaves, stems and roots were measured. The dry mass and the amounts of sodium, and potassium ions were measured in four replicate samples of the mapping population. These data were used to calculate seventeen separate phenotypic parameters. Na^+ and K^+ were assayed by flame emission spectrophotometry (Flame Photometer 410, Corning) after nitric acid extraction (HNO3, 0.5%) of the finely ground dry leaf, stem, and root tissue [48].

4.3. Genetic mapping

A RIL population, named LR5, was derived from the cross between Jemalong A17 (JA17) and F83005.5 (F83) *M. truncatula* lines. The framework genetic map of LR5 was constructed as described by

[49]. The RIL population was genotyped with 70 SSR markers. These SSR markers were identified on the basis of *M. truncatula* physical map (www.medicago.org). A part of the used markers had been previously mapped on the genome of the model legume *M. truncatula* using LR4 framework genetic map [49]. The map covers 669.4 cM with an average interval between markers of 9.56 cM. This map LR5 globally covers the entire genome of the species, the integrated genetic map of *M. truncatula* covering 596 cM (http://www.medicago.org).

4.4. Statistical analysis and QTL mapping

The complete set of data was involved in an analysis of variance (ANOVA), using the Statistical Analysis System (SAS 7.02 Institute, Inc., 1998), to determine the specific effects of genotype (i.e. the RIL) and replication (i.e. the cultivation replication) factors. Estimated environmental variance (σ_e^2) was performed using Proc GLM where replication and genotype were considered as fixed effects, while estimation of genetic variance was calculated using Proc VARCOMP; considering the genotype effect as a random effect. Estimates of the genetic variance component (σ_g^2) and environmental variance (σ_e^2) were used to calculate broad-sense heritabilities (H^2) for all measured traits.

$$H^2 = \sigma_g^2 / \left(\sigma_g^2 + \sigma_e^2/k\right)$$

where k is the number of replicates per line. Phenotypic correlations for all combinations of measured traits for lines in control and 50 mM of NaCl treatments were estimated by computing the Pearson correlation coefficient (r) using the SAS CORR procedure (SAS 7.02 Institute, Inc.). Sensitivity index (SI) in trait expression in response to salt stress treatment was determined as following:

$$\mathrm{SI} = (\mathrm{S}-\mathrm{C}) * 100/\mathrm{C}$$

where S is the value of measured trait in salt treatment and C is the value of the same trait in control conditions.

Proprieties of the trait distribution were estimated by ANOVA analysis over 4 replicates. We measure Kurtosis and Skewness parameters using SPSS software (13.0 for windows).

The software package PlabQTL version 1.2 [50] was used to identify and locate QTL on the linkage map of LR5 by using standard methods as described in its reference manual (utzf@uni-hohenheim.de, http://www.unihohenheim.de/~ipspwww/soft.html). In a first step, putative QTLs involved in the variation of the trait were identified using simple interval mapping (SIM) [51]. Thereafter, composite interval mapping (CIM) was performed on the same data: the closest marker to each local LOD score peak (putative QTL) was used as a cofactor to control the genetic background while testing at a position of the genome. The Log of odds (LOD) significance threshold (2.79 LOD) was determined by a permutation test of 1000 permutations with a 5%-alpha risk [27]. QTL positions were estimated where the LOD score reached its maximum in the region under consideration. The individual QTL effects (R^2) were estimated as the percentage of the variance explained by the QTL conditioned on the background markers. Additive effects (2a) of detected QTL were estimated from CIM results; 2a represents the mean effect of the replacement of both F83 alleles by JA17 alleles at the studied locus.

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